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RNA splicing products formed with isolated fractions from HeLa cells are associated with fast-sedimenting complexes

(RNA processing/adenovirus/β-globin)

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ABSTRACT **Three fractions (designated Ia, Ib, and II) have been isolated from HeLa cell nuclear extracts that are required for splicing of adenovirus and human β-globin RNA transcripts *in vitro*. The incubation of two of the fractions (Ib and II) in the presence of ATP resulted in cleavage of precursor mRNA at the 5′ splice site and formation of the intron–exon lariat. Addition of fraction Ia to the combination of Ib and II resulted in the formation of spliced RNA and the intron lariat. When fraction II was incubated with precursor RNA in the presence of ATP and the resulting products were sedimented through sucrose gradients, a 30S complex was detected that contained precursor RNA. The combination of fractions Ib and II resulted in the production of a 55S complex that contained the 5′ exon as a prominent RNA species. The combination of fractions I (containing Ia and Ib) and II resulted in the formation of the 55S complex and material sedimenting between 40 S and 20 S, in which the predominant RNA species was spliced RNA.**

The elimination of intervening sequences from primary transcripts by RNA splicing is essential for the correct expression of many eukaryotic genes (1). Studies of the kinetics of RNA splicing *in vitro* and characterization of the likely reaction intermediates suggest that RNA splicing proceeds by at least two discrete steps (2–4). Early in the reaction, cleavage of precursor mRNA (pre-mRNA) occurs at the 5′ splice site, and the 5′-terminal phosphate of the intron becomes esterified to the 2′ hydroxyl group of an adenosine residue within the intron, resulting in the formation of an intron–exon lariat structure (4–6). At a later stage, the 5′ exon species and the intron–exon lariat are thought to interact to form spliced RNA, concomitant with the release of the intron in a lariat configuration. The discovery of lariat structures *in vivo* (7–9) lends considerable support to the validity of the pathway described above.

Studies *in vitro* with cell-free extracts of yeast (10) and HeLa cell nuclear extracts (11, 12) suggest that RNA splicing proceeds via the association of pre-mRNA with fast-sedimenting complexes. This suggestion is strengthened by the observation that the formation of these complexes is ATP-dependent (10–12) and is not observed when extracts that have been depleted of small nuclear ribonucleoprotein particles (snRNPs) are used (11, 12), or when transcripts that are incapable of being spliced are used as substrates (10, 11).

In order to gain further insight into the mechanism of this reaction we have concentrated on the isolation of enzymes required for RNA splicing and have reported previously the isolation of two fractions (I and II) from a HeLa cell nuclear extract that were both required for RNA splicing (13). Here we report the separation of fraction I into two components (Ia and Ib) and their ability to form ATP-dependent splicing complexes with pre-mRNA. In the presence of pre-mRNA and ATP, the combination of fractions Ib and II catalyzes cleavage at the 5′ splice site of pre-mRNA and formation of the intron–exon lariat. The 5′ exon and intron–exon lariat RNAs cosediment in sucrose gradients as a 55S complex. The combination of fraction I (containing both fractions Ia and Ib) and fraction II results in the formation of spliced RNA that is released from the 55S complex.

MATERIALS AND METHODS

Materials. SP6 RNA polymerase fraction V (blue dextran sepharose pool) was purified by the method of Butler and Chamberlin (14). Restriction enzymes were obtained from New England Biolabs, Bio-Rex 70 was from Bio-Rad, vanadyl ribonucleoside was from Bethesda Research Laboratories, and all radioisotopes were from New England Nuclear. ^{32}P-labeled 40S ribosomal subunit prepared from *Artemia salina* was the gift of D. Tabarini of this Institute.

***In Vitro* RNA Splicing Reaction.** The use of plasmids pSp64-HβΔ6 and pKT1 for the synthesis of β-globin and adenovirus major late pre-mRNA by SP6 RNA polymerase was as described (13). The specific activity of all adenovirus transcripts used was 120 cpm/fmol of guanine nucleotide. RNA splicing reaction mixtures (0.05 ml) containing 20 mM Hepes·KOH buffer (pH 7.6), 3 mM $MgCl_2$, 2 mM dithiothreitol, 0.4 mM ATP, 20 mM creatine phosphate, 2% PEG 6000, labeled RNA transcripts (as indicated in legends), and various enzyme fractions (as indicated in legends) were incubated at 30°C for 2 hr (unless otherwise indicated). Reactions were terminated by the addition of 0.2 ml of 2% sodium dodecyl sulfate buffer (200 mM Tris·HCl, pH 7.5/25 mM EDTA/300 mM NaCl/2% sodium dodecyl sulfate) and water to 0.4 ml. After extraction with phenol/chloroform, 1:1 (vol/vol) and precipitation with ethanol, the RNA products were dissolved in formamide, analyzed by polyacrylamide/urea gel electrophoresis, and visualized by autoradiography.

Sucrose Gradient Centrifugation Analysis of mRNA Splicing Complexes. Splicing reaction mixtures (0.10 ml) were stopped by quick-freezing in a dry ice/ethanol bath. The samples were then thawed and applied to 5 ml 10–30% sucrose gradients containing 100 mM KCl, 3 mM $MgCl_2$, and 10 mM Hepes·KOH buffer (pH 7.6). Centrifugation was at 48,000 rpm in a Sorvall AH 650 rotor at 4°C for 195 min.

Preparation of Fractions. Fractions I and II were prepared as described previously (13) from a HeLa cell nuclear extract (15). Fraction I (43 ml; 11.3 mg of protein per ml) was dialyzed against 50 mM KCl in buffer A (20 mM Hepes·KOH buffer, pH 7.6/1 mM dithiothreitol/0.1 mM EDTA/10% (vol/vol) glycerol and applied to a Bio-Rex 70 column (5.5 × 2.5 cm) previously equilibrated with 50 mM KCl/buffer A. The material that passed through the column was collected and pooled to yield fraction Ia (30 ml; 2.7 mg of protein per ml).

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Abbreviations: pre-mRNA, precursor mRNA; snRNPs, small nuclear ribonucleoprotein particles.

The column was then eluted with buffer A containing 1 M KCl. The eluate was pooled to yield fraction Ib (18 ml; 7.5 mg of protein per ml) and dialyzed against buffer A containing 0.1 M KCl. Fractions were stored in aliquots at −70°C, and they retained activity under these conditions for at least 2 months.

RESULTS

We demonstrated previously that the HeLa cell nuclear extract capable of splicing mRNA (2) could be separated into two fractions (I and II), which were both required for the splicing of β-globin and adenovirus pre-mRNAs (13). The combination of fractions I and II catalyzed the ATP-dependent formation of five RNA species from β-globin pre-mRNA that migrated with apparent mobilities corresponding to nucleotide lengths of 450, 370, 252, 155, and 140 on 6% polyacrylamide/urea gels (Fig. 1, lane 1). These species were identified as the intron–exon lariat, spliced RNA, an RNA molecule produced by an ATP-dependent aberrant cleavage within the intron, the 5′ exon, and the intron lariat, respectively (4). None of these products was detected when either fraction was incubated alone (13) (Fig. 1, lane 2). Now we report further separation of fraction I into two fractions, Ia and Ib. The combination of fractions Ia, Ib, and II was required for the formation of spliced RNA and all putative intermediates described above (Fig. 1, lanes 5–7). The combination of fractions Ia and II yielded no ATP-dependent products (Fig. 1, lane 4). However, the combination of fractions II and Ib yielded the RNA species with electrophoretic mobilities of 450 and 155 nucleotides, corresponding to the intron–exon lariat and the first exon (Fig. 1, lane 3), although not clearly shown here. The ability of fractions Ib and II to catalyze cleavage at the 5′ splice site and formation of the intron–exon lariat is analyzed in more detail in the next section.

We previously reported that the combination of fractions I and II catalyzed the ATP-dependent formation of two RNA species from adenovirus pre-mRNA that migrated with apparent mobilities corresponding to nucleotide lengths of 94 and 56 on 6% polyacrylamide/urea gels; these species were identified as spliced RNA and the 5′ exon, respectively (13). No attempt was made to analyze putative lariat structures. In the experiments presented here, we utilized 15% polyacrylamide/urea gels, which permit the visualization of lariat structures because they migrate more slowly than does pre-mRNA (3).

Incubation of the adenovirus pKTI transcript with three fractions (Ia, Ib, and II) yielded four ATP-dependent RNA species with electrophoretic mobilities of 430, 255, 94, and 56 nucleotides (as determined by comparison with DNA size markers) when analyzed by 15% polyacrylamide/urea gel electrophoresis (Fig. 2, lanes 7 and 8). The identity of the 94-nucleotide species as spliced RNA was confirmed by hybridization with the M13 single-stranded cDNA derived from the adenovirus major late mRNA (16) and protection from RNase T1 digestion as described (13).

The RNA species with apparent mobilities of 430 and 255 nucleotides were identified as the intron–exon lariat and the intron lariat, respectively, by their anomalous electrophoretic mobilities, by their RNase T1 digestion patterns, and by the observation that they each yielded a trinucleotide upon digestion with P1 nuclease (data not shown). In addition, incubation of these two RNA species with HeLa cytoplasmic extracts containing the lariat debranching activity described by Ruskin and Green (17), which specifically cleaves 2′,5′-phosphodiester bonds at lariat attachment sites, followed by electrophoresis through 15% polyacrylamide/urea gels resulted in their conversion to RNA species with apparent mobilities characteristic of species of 125 and 86 nucleotides. These are the electrophoretic mobilities expected for the intron–exon lariat and intron lariat, respectively, which have been linearized (data not shown).

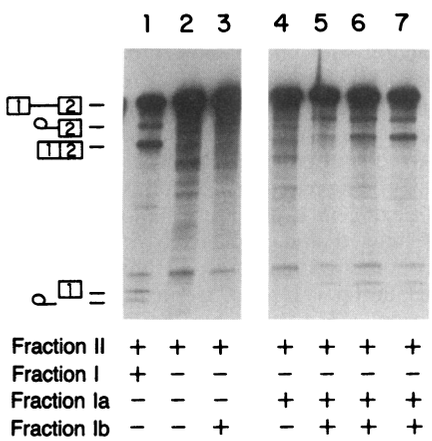

FIG. 1. Fraction Ia is required for *in vitro* splicing of SP6 β-globin RNA. Reaction mixtures contained the SP6 β-globin transcript [1.6 pmol (total guanine nucleotide), 1.35 × 10^5 cpm/pmol], ATP (0.4 mM), creatine phosphate (20 mM), fraction II (46 μg of protein), fraction I (226 μg of protein), and fraction Ib (37 μg of protein) where indicated. Samples were analyzed by 6% polyacrylamide/urea gel electrophoresis. Fraction Ia added in lanes 4–7 was 27, 13, 27, and 54 μg, respectively.

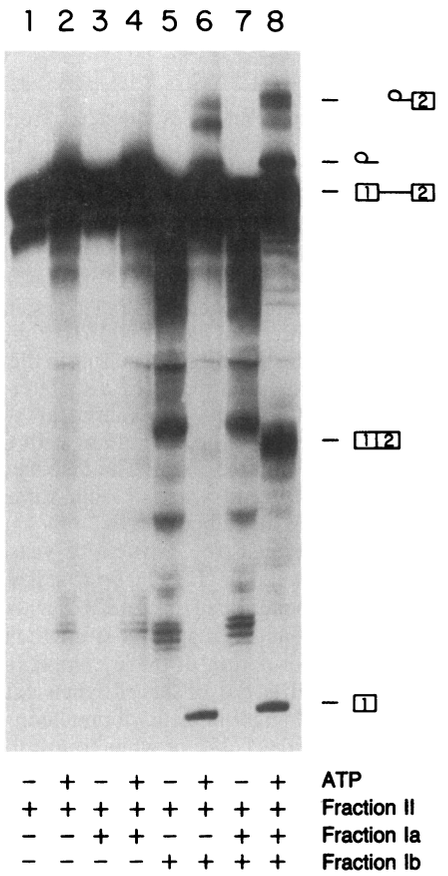

FIG. 2. Fraction Ia is required for *in vitro* splicing of SP6 adenovirus RNA. Reaction mixtures contained SP6 adenovirus transcript [13.6 fmol (ends), 120 cpm/fmol] and, where indicated, ATP (0.4 mM), creatine phosphate (20 mM), fraction II (46 μg of protein), fraction Ia (27 μg of protein), and fraction Ib (37.5 μg of protein). Samples were analyzed by 15% polyacrylamide/urea gel electrophoresis. Lanes 1–8 show combinations of fractions II, Ia, and Ib in the presence or absence of ATP and creatine phosphate.

Incubation of fraction II alone (Fig. 2, lanes 1 and 2) or the combination of fractions Ia and II (Fig. 2, lanes 3 and 4) yielded no ATP-dependent RNA species involved in splicing. The combination of fractions Ib and II resulted in the production of the 430- and 56-nucleotide species, which were identified as the intron–exon lariat and the first exon. Under these conditions, no spliced RNA was formed (Fig. 2, lanes 5 and 6).

The rate of formation of various RNA species in the presence of ATP and fractions Ib and II (Fig. 3*A*, lanes 1–5) or fractions Ia, Ib, and II (Fig. 3*A*, lanes 6–10) was examined. Spliced RNA was not detected when fractions Ib and II were combined (Fig. 3*A*, lanes 1–5). When fractions Ia, Ib, and II were combined, spliced RNA was formed after 60 min of incubation (Fig. 3*A*, lanes 6–10). Quantitation of the amount of spliced RNA formed (after gel excision and liquid scintillation spectroscopy) revealed that 15% of the input pre-mRNA had been converted to spliced RNA after 4 hr (Fig. 3*B*).

The amount of spliced RNA formed by the purified fractions was compared to the amount formed by the HeLa cell nuclear extract. The combination of fractions Ia (27 μg of protein), Ib (37 μg of protein), and II (46 μg of protein) yielded 0.73 fmol of spliced RNA in 2 hr at 30°C, representing 10% of the input adenovirus pre-mRNA. Under the same conditions, the combination of fractions I (226 μg of protein) and II (46 μg of protein) yielded 1.6 fmol of spliced RNA (20% of the input pre-mRNA), and the nuclear extract (300 μg of protein) yielded 1.0 fmol of spliced RNA (12% of the input pre-mRNA). Addition of increasing amounts of fraction Ib resulted in increased cleavage at the 5′ splice site; however, due to an exoribonuclease present in this fraction, the intron–exon lariat was extensively degraded, and less spliced RNA was produced. For this reason, the amount of fraction Ib used in these reactions was limiting and probably accounted for the lower yield of spliced RNA observed.

The amount of cleavage at the 5′ splice site that had occurred in the absence (Fig. 3*A*, lanes 1–5) and in the presence (Fig. 3*A*, lanes 6–10) of fraction Ia was determined by measuring the amount of free 5′ exon observed and the 5′ exon content of spliced RNA. Incubation of fractions Ia, Ib, and II for 1.0, 2.0, and 4.0 hr resulted in 5%, 11%, and 15% cleavage at the 5′ splice site. In contrast, incubation of fractions Ib and II for 1.0, 2.0, and 4.0 hr resulted in 1%, 4%, and 9% cleavage at the 5′ splice site. Therefore, the addition of fraction Ia to the combination of fractions Ib and II results in increased cleavage at the 5′ splice site as well as the production of spliced RNA.

The amount of intron–exon lariat could not be quantitated because of digestion of this species by a 3′ exonuclease activity that is present in fraction Ib (Fig. 3*A*, lanes 1–5). We believe that the RNA species with electrophoretic mobility similar to the intron lariat produced by incubation with fractions II and Ib (Fig. 3*A*, lanes 1–5, and Fig. 2, lane 6) was formed by digestion of the intron–exon lariat (to a specific site) by this 3′ exonuclease. This product is probably not formed by endonucleolytic cleavage at the 3′ splice site, since the 3′ exon species (38 nucleotides in length) has not been observed. In addition, the intron lariat was not detected when β-globin transcript was incubated with fractions Ib and II.

Properties of the Three Fractions. Reactions containing fractions Ia, Ib, and II displayed the same requirement for ATP and creatine phosphate observed with the nuclear extract and with the combination of fractions I and II (13). Addition of creatine phosphokinase did not stimulate the reaction nor did it substitute for any of the fractions. Addition of vanadyl ribonucleoside at concentrations of 40, 80, and 400 μM to the reaction mixtures resulted in 5%, 64%, and 100% inhibition, respectively, of the formation of all RNA splicing products, suggesting that inhibition occurred at an early step in the reaction.

The activity in fraction Ia was relatively unaffected by treatment with *N*-ethylmaleimide (5 mM) and by heating at 55°C for 10 min but was inactivated by heating at 65°C for 10 min (Table 1). In contrast, the activity in fraction Ib was abolished by treatment with *N*-ethylmaleimide (5 mM) and by heating at 55°C for 10 min (Table 1). The activity in fraction II, as described previously (13), was abolished by *N*-ethylmaleimide (5 mM) and heat (65°C for 30 min).

Analysis of Macromolecular Complexes Formed by Fractions Ia, Ib, and II. Earlier studies on mRNA splicing *in vitro* suggested that the first step in the reaction might be the assembly of a ribonucleoprotein complex (6). The existence of such complexes has recently been observed in both yeast and mammalian systems (10–12). Therefore, it was of interest to establish which, if any, of the protein fractions described above was capable of forming a complex with precursor RNA. Incubation (2 hr at 30°C) of the adenovirus pre-mRNA with fractions I and II followed by sucrose gradient sedimentation analysis revealed the ATP-dependent formation of

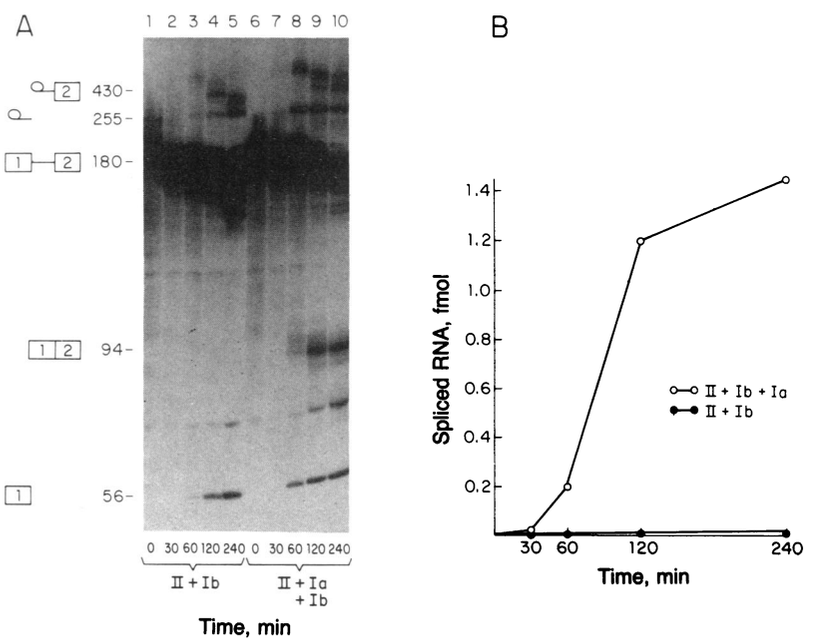

FIG. 3. Kinetics of the splicing reaction. Reaction mixtures contained SP6 adenovirus transcript [13.6 fmol (ends), 120 cpm/fmol], ATP (0.4 mM), creatine phosphate (20 mM), fraction II (46 μg of protein), fraction Ib (37.5 μg of protein), and fraction Ia (27 μg of protein) as indicated. (*A*) In lanes 1–5, fractions Ib and II were combined and incubated for the indicated time; in lanes 6–10, fractions Ia, Ib, and II were combined and incubated as indicated. (*B*) Spliced RNA from *A* was quantitated by excision of gel fragments followed by liquid scintillation spectroscopy.

Table 1. The effect of heat and *N*-ethylmaleimide on fractions Ia and Ib

Treatment	Activity of fractions treated	
	Ia*	Ib†
None	2.15	1.18
N-ethylmaleimide (5 mM)	1.24	<0.01
10 min at 37°C	1.80	0.60
10 min at 45°C	1.13	0.45
10 min at 55°C	0.83	0.02
10 min at 65°C	<0.01	—

All reaction mixtures containing fraction II (46 μg of protein), fraction Ib (37.5 μg of protein), SP6 adenovirus transcript (40 fmol, 120 cpm/fmol), ATP (0.4 mM) and creatine phosphate (20 mM) were incubated for 2 hr at 30°C.

*Assay of fraction Ia: in reactions including fraction Ia (27 μg of protein), spliced RNA (fmol) produced in 2 hr at 30°C was measured.

†Assay of fraction Ib: the quantity of 5′ exon (fmol) produced in 2 hr at 30°C was measured.

material much larger than the 6S precursor RNA (Fig. 4*A*). The sedimentation coefficients of the two main peaks of radioactivity were determined to be 55 S and 20 S by using a 40S ribosomal subunit and 6S pre-mRNA as markers. The specificity of this interaction was investigated by using a precursor RNA that could not undergo cleavage at the 5′ splice site, the first observable step in mRNA splicing. A truncated adenovirus pre-mRNA was synthesized from plasmid pKT1 digested with the restriction enzyme *Hha* I. This pre-mRNA contained the first exon and only 25 nucleotides of the intron. The essential role of 3′ intron sequences in the cleavage at the 5′ splice site has been documented (18). Incubation of this pre-mRNA with fractions I and II did not result in cleavage at the 5′ splice site (data not shown) and, therefore, provided a useful control transcript to ascertain RNA–protein interactions that are involved in mRNA splicing. Incubation of the truncated pre-mRNA with fractions I and II did not result in the formation of the fast-sedimenting complexes described above (Fig. 4*A*). This result, taken in conjunction with the stringent ATP requirement, suggests that the fast-sedimenting material is a product of the RNA splicing system and not merely the nonspecific binding of the RNA to large components in the reaction mixture.

The structure of the RNA found in these ATP-dependent complexes was analyzed by 15% polyacrylamide/urea gel electrophoresis. Precursor RNA was found in almost all fractions but was visibly concentrated in the 55S peak (Fig. 4*B*). Spliced RNA was also found in the 55S peak but was predominant in an area between 40 and 20 S. The intron lariat was present in the 55S peak, but the majority of this species was found between 55 and 40 S. In contrast, the 5′ exon species was located almost exclusively in the 55S peak. Although not clearly shown here, in other experiments the intron–exon lariat species was also found to be exclusively associated with the 55S complex albeit present in a low amount.

A time course of complex formation (data not shown) indicated that the 55S complex was formed after 30 min of incubation. There was no apparent increase in the amount of radioactivity associated with the 55S complex upon further incubation for 60 and 120 min. Gel analysis of individual fractions revealed that, although the amount of 5′ exon associated with the 55S complex remained constant throughout this time period, the amount of spliced RNA in the 20–40 S area both markedly increased and progressively sedimented more slowly.

We next investigated which, if any, of the fractions when incubated alone was capable of forming a complex with pre-mRNA. Neither fraction I nor its derivative fractions Ia and Ib (data not shown) formed any discernible (>10S) complex with the adenovirus pre-mRNA (Fig. 5*A*). On the other hand, fraction II, the micrococcal nuclease-sensitive fraction, did form a 30S complex, which on analysis was found to contain precursor RNA (Fig. 5*A Inset*). This interaction is likely to be involved in mRNA splicing because it required ATP (Fig. 5*A*) and was not manifested with the truncated precursor (data not shown).

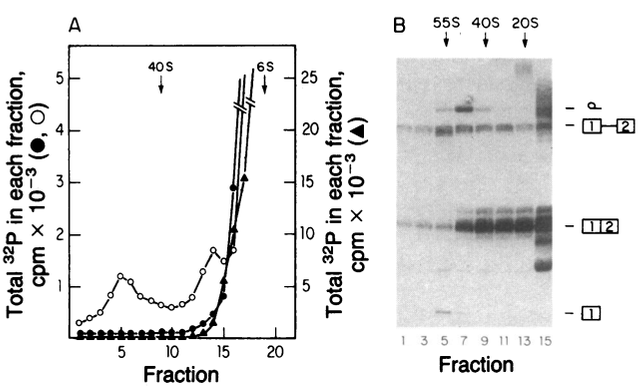

FIG. 4. Sedimentation analysis of macromolecular complexes formed during the splicing of adenovirus pre-mRNA. (*A*) Fraction I (452 μg of protein) and fraction II (92 μg of protein) were incubated with adenovirus pre-mRNA [84 fmol (ends), 120 cpm/fmol] in the presence (○) or absence (●) of ATP (0.4 mM) and creatine phosphate (20 mM). The truncated pre-mRNA (▲) was also incubated with ATP (0.4 mM) and creatine phosphate (20 mM) under the same conditions. The resulting macromolecular complexes were analyzed by sucrose gradient centrifugation as described. The indicated 40S and 6S markers refer to the sedimentation positions of the 40S ribosomal subunit and adenovirus pre-mRNA, respectively. (*B*) Radioactive material in the indicated gradient fractions was extracted with phenol/chloroform, precipitated by addition of ethanol, and analyzed by 15% polyacrylamide/urea gel electrophoresis. The various RNA structures are indicated schematically on the right.

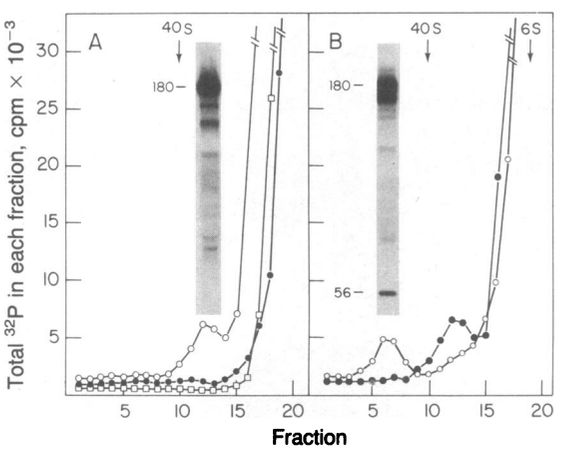

FIG. 5. The formation of a 30S complex by fraction II and a 55-S complex by fractions II plus Ib. (*A*) Fraction I (□) or fraction II (○) were incubated (2 hr at 30°C) with pre-mRNA [84 fmol (ends), 120 cpm/fmol] in the presence of ATP (0.4 mM) and creatine phosphate (20 mM), and the resulting complexes were analyzed by sucrose gradient centrifugation. Fraction II also was incubated in the absence of ATP and creatine phosphate (●). (*B*) Fraction II and fraction Ia (●) or fraction II and fraction Ib (○) were incubated (2 hr at 30°C) with adenovirus pre-mRNA [84 fmol (ends), 120 cpm/fmol], and the resulting complexes were analyzed by sucrose gradient centrifugation. (*Insets*) RNA compositions of fractions 12 (*A*) and 6 (*B*). Amounts of fractions used were as follows: fraction I, 452 μg; fraction II, 92 μg; fraction Ia, 54 μg; and fraction Ib, 75 μg.

We next determined which combination of fractions could yield the 55S complex. Fig. 5*B* shows that the addition of fraction Ia to fraction II yielded only the 30S complex. In contrast, incubation of fractions Ib and II yielded a 55S complex, which on gel analysis was found to contain the 5′ exon and to a lesser extent the intron–exon lariat species. Since the combination of fractions Ib and II yielded the first observable event in mRNA splicing (see above) this strengthens the conclusion that the 55S complex is involved in the splicing of RNA.

DISCUSSION

We previously reported the isolation and characterization of two fractions (I and II) from nuclear extracts of Hela cells that were required for ATP-dependent splicing of both adenovirus and β-globin pre-mRNAs. In this report we described the further separation of fraction I into two components (Ia and Ib), which have been distinguished on the basis of their sensitivities to *N*-ethylmaleimide and heat. The combination of fractions Ia, Ib, and II was required for the production of spliced RNA and the intron lariat. However, two of these fractions (II and Ib) were sufficient for cleavage at the 5′ splice site and intron–exon lariat formation, apparently uncoupling the proposed two-stage splicing mechanism. Quantitation of the reaction products revealed that when fractions Ib and II were combined, the amount of cleavage at the 5′ splice site was less than the amount of cleavage occurring when fractions Ia, Ib, and II were combined. These results suggest that the addition of fraction Ia (that results in the formation of spliced RNA) enhances further cleavage at the 5′ splice site of pre-mRNA. We have reported that fraction Ib was added in limiting amounts to the splicing reactions because of the presence of an exoribonuclease activity in this fraction that degraded the intron–exon lariat. It is unlikely that the amount of 5′ exon observed in the absence of Ia was decreased by this exonuclease because the time-dependent degradation of this species was never observed.

We also demonstrated that RNA splicing products formed with the fractionated system were associated with fast-sedimenting complexes. Our observations are similar to those of Frendewey and Keller (12) and Grabowski *et al.* (11) in that putative splicing intermediates were found to be associated with a large complex and that pre-mRNA was found to be associated with a smaller complex. These groups have provided evidence that the smaller complex was formed as a precursor to a larger complex (12) and that complex formation required the snRNPs (11, 12).

In this report we have established which fractions are required for complex formation. Incubation of fraction II with pre-mRNA resulted in the ATP-dependent formation of a 30S complex that contained pre-mRNA. Since we know that fraction II is sensitive to micrococcal nuclease (13), we believe that RNA components may be involved in the formation of this complex. Attractive candidates for these RNA components are the snRNPs as suggested by other investigators (11, 12). Incubation of fractions II and Ib with pre-mRNA in the presence of ATP resulted in the formation of a 55S complex, which contained the 5′ exon and the intron–exon lariat as well as pre-mRNA.

We speculate that 30S complex formation is the first step in the splicing reaction and may be a precursor to the 55S complex. Our argument is strengthened by the use of a mutant pre-mRNA in which the 3′ splice junction has been changed from CAG to CAC. This mutant undergoes less cleavage at the 5′ splice site and is not capable of yielding spliced RNA (data not shown). When this mutant is incubated with fractions II and Ib, very little 55S complex (11% of wild type) is formed, and predominantly 30S material is formed (data not shown). At present we cannot ascertain whether the component(s) in fraction Ib bind directly to the pre-mRNA or to the 30S complex, nor can we establish whether ATP is required for the proposed transition from 30S to 55S. The isolation of an active 30S complex that could be reincubated with fraction Ib to yield the putative intermediates would be likely to resolve these questions.

We also demonstrated that when fraction I (which contains fractions Ia and Ib) was incubated with fraction II, the 55S complex was formed, which contained the putative intermediates as well as spliced RNA. However, the majority of the spliced RNA produced under these conditions sedimented between 40 and 20 S. Thus, it appeared that spliced RNA was released from the 55S complex. The amount of the 5′ exon contained within the 55S complex formed with fractions I and II did not increase with time, although increasing amounts of spliced RNA were released.

Our observations suggest, therefore, a model for RNA splicing in which the first step is the ATP-dependent association of pre-mRNA with snRNPs and as yet unidentified proteins to form a 30S complex. In the presence of the appropriate splicing factors (fraction Ib), a 55S complex is formed that produces the putative intermediates suitably juxtaposed for ligation of exon 1 to exon 2. In the presence of fraction Ia, spliced RNA is formed, resulting in the dissociation of the 55S complex. The components of the 55S complex are now available to reassemble on a new pre-mRNA molecule resulting in cleavage at the 5′ splice site.

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